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Cotyledonary responses to maternal selenium and dietary restriction may influence alterations in fetal weight and fetal liver glycogen in sheep☆

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ABSTRACT

To examine the effects of maternal supranutritional selenium (Se) and nutrient restriction during mid and late gestation on placental characteristics and fetal liver glycogen, ewes received either adequate Se (ASe) or high Se (HSe) prior to breeding. On d 64 of gestation, ASe and HSe ewes remained at 100% of requirements (controls; CON) or were restricted (RES; 60% of requirements). On d 135 of gestation, fetal weight ($P \leq 0.08$) was greatest in both HSe and CON ewes. Placentome number, mass, and caruncular and cotyledonary weight were not different ($P \geq 0.17$) among treatments. Fetal mass:placental mass ratio was less ($P = 0.06$) in RES compared to CON ewes. Compared to ASe, HSe exhibited increased ($P \leq 0.08$) cellular proliferation and DNA concentration and decreased ($P = 0.07$) cellular size in cotyledonary tissue. Nutritional restriction decreased ($P \leq 0.08$) cotyledonary protein concentration and cellular size. *VEGF receptor 1* (*Flt*) mRNA in cotyledonary tissue was greater in HSe compared with ASe ewes ($P = 0.06$) and in RES compared with CON ewes ($P = 0.08$). There was no effect of diet on caruncular growth variables ($P \geq 0.13$) or on placental vascularity ($P \geq 0.11$). Progesterone was greater ($P \leq 0.08$) in ASe–RES ewes compared to all groups at d 90 and ASe–CON and HSe–CON at d 104. Although fetal glucose and cortisol concentrations were not affected by diet, fetal liver glycogen was greater ($P = 0.04$) in ASe–RES compared to ASe–CON and HSe–RES ewes with HSe–CON being intermediate. Both Se and nutritional plane may impact placental function and fetal growth, as fetal weight and liver glycogen are altered despite similar placental vascularity measurements.

1. Introduction

Intrauterine growth restriction (IUGR) is a major concern for the livestock industry because fetal growth restriction leads to negative impacts later in life on animal performance such as postnatal growth, body composition, and reproductive performance (reviewed by Wu et al., 2006). For grazing ruminants, poor forage quality results in inadequate nutrition to meet gestational demands (Hoaglund et al., 1992; Huston et al., 1993). Similarly, IUGR results in fetal and infant mortality and morbidity in humans (Bernstein et al., 2000), as well as impacting health

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later in life. In human preeclampsia, fetuses are normally delivered early, are low in birth weight, and may have further complications later in life (reviewed by Sibai et al., 2005). Recently, Mistry et al. (2008) have demonstrated that women suffering from preeclampsia have reduced concentrations of selenium (Se) in umbilical venous blood compared to normal pregnancies. When comparing trace mineral levels in normal and IUGR babies born at similar ages, IUGR babies have a greater Se concentration in umbilical arterial blood and their placental tissues compared to control babies (Osada et al., 2002).

Global nutrient restriction during mid to late pregnancy in the ewe can reduce fetal weight at term (Faichney and White, 1987). Maternal nutrient restriction during mid to late gestation decreased fetal weight compared to adequately fed controls whereas ewes receiving supranutritional levels (levels above NRC recommendations) of Se had increased fetal weight compared to ewes receiving adequate levels of Se (Reed et al., 2007). Further, fetuses from nutrient restricted ewes had decreased liver and perirenal fat mass, and decreased carcass weight (Reed et al., 2007), indicating that fetal energy stores may further be affected by maternal diet.

The combined effects of maternal nutrient restriction and supranutritional Se on placental development and fetal nutrient stores are unknown. We hypothesize that the reduction in fetal mass by nutrient restriction is due to a reduction in placental size and/or vascularity, while Se supplementation would increase placental size and/or vascularity which led to the increased fetal weight. The objectives of the present study were to determine how supranutritional Se throughout pregnancy and/or nutrient restriction during mid to late gestation affect circulating fetal glucose and cortisol concentrations, amounts of fetal liver glycogen, and placental growth.

2. Materials and methods

2.1. Animals and treatments

All procedures were approved by the North Dakota State University and USDA-ARS Animal Care and Use Committees. Twenty-one days prior to breeding, Targhee-Columbia crossbred, primiparous ewe lambs (8.5 ± 0.5 mo of age) were placed in group-fed pens at the U.S. Sheep Experiment Station in Dubois, ID and were assigned randomly to Se treatments [adequate Se (ASe) and high Se (HSe)], which continued until the end of the study (Reed et al., 2007). Briefly, the ASe ewes received 6 μg of Se per kilogram body weight per day, and the high-Se (HSe) ewes received 80 μg of Se per kilogram body weight per day. Ewes received a basal diet plus a Se supplement (ASe ewes received a pelleted supplement containing no added Se [0.3 ppm Se], HSe ewes received a pelleted supplement containing Se-enriched yeast [43.2 ppm Se; Sel-Plex; Alltech Inc., Nicholasville, KY]). On d 50 of gestation pregnant ewes ($n = 36$) were shipped to the Animal Nutrition and Physiology Center at North Dakota State University and were housed individually in 0.91 m × 1.20 m pens in a temperature controlled (12 °C) and ventilated facility with lighting automatically timed to mimic day length at the latitude of Fargo, ND. Within each Se treatment, ewes were assigned to one of two nutritional treatments [100% (CON) vs. 60% (RES) of the requirements for gestating ewe lambs; NRC, 1985] on d 64 of gestation. This results in four treatments with the treatments arranged as a 2×2 factorial array. The main effects evaluated were dietary Se (ASe and HSe) and plane of nutrition (CON and RES) resulting in four treatments: ASe–CON ($n = 8$), ASe–RES ($n = 10$), HSe–CON ($n = 10$), and HSe–RES ($n = 8$). As previously reported by Reed et al. (2007), ewes were fed once daily, with free access to water and trace-mineralized salt containing no added Se (American Stockman, Overland Park, KS), and the diets consisted of chopped alfalfa hay top-dressed with whole corn and the pelleted supplements. Body weight was measured on d 59 and 73 of gestation, and every 7 d thereafter until the end of the study, and the diets were adjusted accordingly. Jugular venous blood samples were taken from the ewes every 14 d.

2.2. Tissue collection procedures

One ewe from each treatment was assigned randomly to each of nine slaughter days, and the average day of gestation at slaughter was 135 ± 5 d (mean ± range). One hour prior to slaughter, the ewes were weighed to obtain a final body weight, a jugular venous blood sample was collected and placed in a nonheparinized tube on ice for 2 h prior to centrifugation, and the ewes were injected via the jugular vein with 5-bromo-2-deoxy-uridine (BrdU; 5 mg/kg BW). At slaughter, the ewes were stunned with a captive bolt gun (Supercash Mark 2, Acceles and Shelvoke Ltd., England), exsanguinated, and the gravid uterus was dissected cranial to the cervix and weighed. The uterus was opened along the antimesometrial side, the fetus was removed from the amnion still attached to the umbilical cord, and fetal blood was collected via cardiac puncture using a 20-gauge needle and 10-mL syringe. The fetal blood was put into a nonheparinized tube and placed on ice for 2 h prior to centrifugation. Thereafter, the umbilical cord was ligated, and the fetus was removed and weighed. The fetal liver was removed, weighed, snap frozen in supercooled isopentane (submerged in liquid nitrogen) and stored at −80 °C (Reynolds et al., 1990a; Reynolds and Redmer, 1992).

Immediately after the fetus was removed, several placentomes were dissected from the uterus and weighed. The caruncular and cotyledonary tissues were separated, weighed, snap frozen, and stored at −80 °C. Next, a portion of the uterine tract was perfusion-fixed by methods of Borowicz et al. (2007). Briefly, the main uterine arterial and the main umbilical arterial branches were catheterized and several placentomes were perfused first with PBS, followed by Carnoy's solution (a nonaldehyde-based fixative composed of 60% EtOH, 30% acetic acid, 10% chloroform), then with PBS, and, finally with a latex vascular casting resin (Microfil MV-132, 4 mL of latex compound with 5 mL of diluent, all from Flow Tech, Inc., Carver, MA) and then immersion-fixed in Carnoy's fixative for 24 h. The remaining placentomes were then dissected, weighed, and the average placentome weight was recorded.

2.3. Measurement of placental vascularity

After fixation, the placentomes were embedded in paraffin using standard procedures. The perfused placental tissue blocks were sectioned at 4 μm, mounted on glass slides, and stained using periodic acid Schiff's staining procedures (Luna, 1968) to provide contrast to the vascular tissue, as previously described (Borowicz et al., 2007; Vonnahme et al., 2007).

Photomicrographs were taken at 40× and 60× magnification for caruncular and cotyledonary tissues, respectively, using a Nikon DSM 1200 digital camera (Fryer Company, Inc., Chicago, IL). Vascularity ($n = 4$ ewes per treatment) was then determined by image analysis (Image-Pro Plus, version 5.0, Media Cybernetics, Houston, TX) (Borowicz et al., 2007; Vonnahme et al., 2007). Briefly, for each ewe, 10 areas per placentome were analyzed for tissue area, shrinkage area (i.e., the effect of fixation, which was subtracted from the tissue area), cross-sectional capillary area density (CAD, total capillary area as a proportion of tissue area), capillary number density (CND, total number of capillaries per unit of tissue area), and capillary surface density (CSD, total capillary circumference per unit of tissue area) which is proportional to capillary surface area (Borowicz et al., 2007; Vonnahme et al., 2007). The average cross-sectional area per capillary (APC), which represents the average cross-sectional size of the capillaries, was calculated for caruncular and cotyledonary tissue by dividing CAD by CND.

2.4. Measurement of placentome cellular proliferation

The BrdU was used as a marker of cellular proliferation in the tissue sections (Jablonka-Shariff et al., 1993; Jin et al., 1994). Prepared placental tissue samples from all ewes were incubated with anti-BrdU formalin grade, mouse IgG, monoclonal antibody (Clone BMC, Roche Diagnostics, Indianapolis, IN) at a 1:200 dilution (9 μL/1.8 mL of blocking buffer). Primary antibody was detected using a biotinylated secondary antibody (Vectastain, Vector Laboratories, Burlingame, CA), Avidin DH: biotinylated secondary antibody complex (Vector Laboratories), and 3,3′-diaminobenzidine (DAB; Vector Laboratories) as a substrate. Positive BrdU staining identified cells undergoing proliferation in the S stage of the cell cycle. Hematoxylin (EMD Chemicals, Inc., Gibbstown, NJ) was used to counterstain the nondividing nuclei and the periodic acid Schiff's staining procedure (Luna, 1968) was used to highlight other structures present within the placental tissue cross-section. Photomicrographs were taken at 40× magnification using a Nikon DSM 1200 digital camera (Fryer Company, Inc.). Cellular proliferation was quantified using Image-Pro Plus software (version 5.0, Media Cybernetics). The percentage of proliferating cells was estimated by dividing the number of DAB-stained nuclei by the total number of nuclei present within the area of tissue analyzed.

2.5. Cellularity estimates

Freshly thawed tissue samples (0.5 g) were homogenized in 0.05 M Tris aminomethane, 2.0 M sodium chloride, and 2 mM EDTA buffer (pH 7.4) using a Polytron with a PT-10 s probe (Brinkmann, Westbury, NY). The caruncular and cotyledonary samples were analyzed for DNA, RNA, and protein concentration, and the fetal liver samples were analyzed for DNA concentration. The DNA and RNA analysis was done using the diphenylamine and orcinol procedures, respectively (Johnson et al., 1997; Reynolds et al., 1990a). Protein in tissue homogenates was determined with Coomassie brilliant blue G (Bradford, 1976) with bovine serum albumin (FractionV, Sigma Chemical) as the standard (Johnson et al., 1997). The prepared samples were analyzed with a spectrophotometer (Beckman DU 640, Beckman Coulter, Inc.) and were assessed against concentration curves of known standards. The concentration of DNA was used as an index of hyperplasia, and protein:DNA and RNA:DNA ratios were used as indexes of hypertrophy and potential cellular activity, respectively (Scheaffer et al., 2004; Soto-Navarro et al., 2004; Swanson et al., 2000).

2.6. Quantification of angiogenic and vasoactive factors

Vascular endothelial growth factor (VEGF), its receptors VEGFR1 (Flt) and VEGFR2 (KDR), endothelial nitric oxide synthase (eNOS), and soluble guanylate cyclase (sGC) are important angiogenic and vasoactive factors affecting placental vascular development (Borowicz et al., 2007; Redmer et al., 2005; Vonnahme et al., 2007, 2008a,b). Caruncular and cotyledonary RNA was extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH), and total RNA concentration was determined by capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to quantify amounts of mRNA for VEGF, Flt, KDR, eNOS, and sGC using methods described (Borowicz et al., 2007; Redmer et al., 2004, 2005; Vonnahme et al., 2006), with the modification that a multiplex reaction was performed after 18S mRNA was added to each well to serve as an internal control (Vonnahme et al., 2008a). The ratio of the gene of interest to the 18S RNA was used for quantifying the gene expression.

2.7. Hormone and glucose analysis

Maternal and fetal blood samples were centrifuged (Beckman Coulter, Fullerton, CA) for 30 min at 1500 × *g*, and the serum was frozen at −20 °C until it was assayed. The ewe serum samples (50 μL) were assayed for progesterone concentrations (Galbreath et al., 2008). Maternal serum was used in duplicate in three assays by the chemiluminescence immunoassay (Immulite 1000, Siemens, Los Angeles, CA). Within each assay, low-, medium-, and high-progesterone pools were assayed in duplicate. The intraassay and interassay coefficient of variance (CV) were 6.5% and 14.0% respectively.

Fetal serum samples were assayed for cortisol concentration in duplicate one assay by the chemiluminescence immunoassay (Immulite 1000, Siemens). Within each assay, lesser-, medium-, and greater-cortisol pools were assayed in duplicate. The intraassay CV was 7.5%.

Fetal serum glucose concentration was determined by a colorimetric assay. Five microliters of serum sample, 5 μL of each standard (8 standards total, ranging from 17.65 to 300.00 μg/dL), 5 μL of Accutrol serum (used for control; Sigma, St. Louis, MO), and 5 μL of 18 MΩ water (used for blank) were pipetted in duplicate into a 96-well plate (Costar EIA/RIA plate #3369, Corning Inc., Corning, NY). Next, 250 μL of Infinity Glucose Reagent (TR15421, Thermo Electron Corporation, Pittsburgh, PA) was pipetted into each well. Two plates were used and each was incubated in a plate reader at 37 °C for 15 min and absorbance was measured at 340 nm (Spectra Max 340, Fullerton, CA). The intraassay and interassay CV were 4.5% and 6.5%, respectively.

2.8. Glycogen analysis

Fetal liver glycogen was determined by a colorimetric assay using glucose as the standard. Tissue samples (200 mg) were homogenized with 2.5 mL of sterile saline (0.9% NaCl) and added to a scintillation vial (Beckman Mini Poly-Q). After centrifugation, 100 μL of the supernatant was transferred to a microcentrifuge tube in duplicate. To one of the duplicates, 100 μL of 50 mM pyridine acetate with 0.2% sodium azide (Sigma) and 100 μL of amyloglucosidase solution (66.61 IU/mL, Sigma) were added. Microcentrifuge tubes were incubated in a 37 °C water bath for 2 h. Tubes without any added solution (i.e., pyridine acetate and amyloglucosidase; used to determine the amount of free glucose in the samples) were stored at 20 °C for 2 h after which time all tubes were vortexed. Then, 7.5 μL of the incubated samples were placed into wells of a microtiter plate (Costar EIA/RIA plate #3369, Corning Inc.) in quadruplicate, 7.5 μL of the unincubated samples were placed into wells in duplicate, and 7.5 μL of the standards were placed into the wells in triplicate. Next, 200 μL of cold glucose infinity reagent (TR15421, Thermo Electron Corporation) was added to the wells. Four plates were used, and each plate was incubated in a plate reader at 37 °C for 20 min, and the absorbance was measured at 340 nm (Spectra Max 340). The intraassay and interassay CV were 1.65% and 1.64%, respectively. Liver glycogen per cell was calculated by dividing fetal glycogen concentration by fetal liver DNA concentration. Total liver glycogen was calculated by multiplying fetal liver glycogen concentration by liver weight.

2.9. Statistics

The data were analyzed as a completely randomized design with a 2×2 factorial arrangement of treatments using the GLM procedure (SAS Inst. Inc., Cary, NC). Ewe body weight and maternal progesterone concentration were analyzed using PROC MIXED, because the data were taken over time. A covariate for fetal number was used in the model as ewe lambs had either single or multiple fetuses. If fetal number was significant ($P \leq 0.10$), it was retained in the model, and if not, it was dropped. The model contained the effects of Se (ASe and HSe), amount of nutrition (CON and RES), day (where appropriate), and all the interactions. When interactions were significant ($P \leq 0.10$), means were separated by using the least significant difference procedure. Main effects were considered significant when $P \leq 0.10$. Least squares means ± pooled standard errors of the mean are presented.

3. Results

3.1. Gross weights

A total of 13, 16, 15, and 10 fetuses were collected from the ASe–CON, ASe–RES, HSe–CON, and HSe–RES ewes, respectively. There was a Se × nutritional amount × day interaction on ewe body weight ($P = 0.001$; Fig. 1). Prior to implementation of dietary restriction on d 64, all ewes had similar ($P \geq 0.27$) body weight. From d 87 until slaughter, the CON ewes were heavier ($P \leq 0.10$) than RES ewes. There were no Se × nutritional amount interactions on fetal

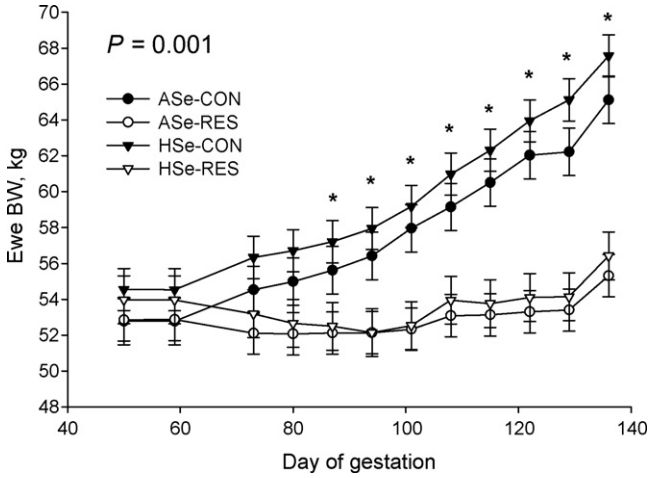

Fig. 1. Effect of dietary selenium (Se) × nutritional amount × day on ewe body weight throughout gestation. ASe = adequate Se ewes (7.4 μg of Se/kg body weight); HSe = high Se ewes (81.5 μg of Se/kg body weight); CON = control ewes fed at 100% requirements; RES = ewes fed at 60% of CON. (*) Both ASe–CON and HSe–CON are statistically different from ASe–RES and HSe–RES ($P \leq 0.10$).

Table 1
Effect of dietary selenium (Se) and amount of nutrition on total fetal mass, average fetal mass, placentome number, total placentome mass, average placentome mass, caruncular wt, cotyledonary wt, and fetal mass:placental mass.

Item	Selenium[a]		Nutrition[b]		SE	*P*-value		
	ASe	HSe	CON	RES		Se	Nut	Se × Nut
Initial ewe mass, kg	52.878	54.20	53.70	53.37	0.81	0.27	0.78	0.73
Final ewe mass, kg	60.19	62.03	66.33	55.89	1.04	0.22	0.0001	0.66
Total fetal mass[c], kg	5.42	5.79	5.97	5.24	0.19	0.19	0.01	0.37
Average fetal mass, kg	3.63	4.05	4.05	3.62	0.17	0.08	0.07	0.54
Placentome number	87.94	95.87	93.05	90.77	4.78	0.26	0.74	0.20
Total placentome mass[d], g	496.00	532.33	524.66	503.67	22.35	0.27	0.51	0.85
Average placentome mass, g	6.08	5.59	5.76	5.91	0.40	0.40	0.80	0.17
Caruncular wt, g	91.65	97.63	99.55	89.73	6.45	0.33	0.46	0.33
Cotyledonary wt, g	300.64	322.43	325.57	297.50	18.80	0.24	0.50	0.97
Fetal mass:placental mass[e]	10.98	10.98	11.53	10.43	0.40	0.99	0.06	0.31

[a] ASe = 6 μg of Se/kg BW (no added Se); HSe = 80 μg of Se/kg BW (HSe).
[b] CON = control ewes fed at 100% of the NRC (1985) requirements; RES = ewes fed to 60% of CON.
[c] Total fetal mass = total fetal mass per ewe.
[d] Total placentome mass = total placentome mass per ewe.
[e] Total fetal mass divided by total placentome mass.

mass or placental measurements (Table 1). Fetal mass was less ($P = 0.08$) in the ASe ewes compared to the HSe ewes. Nutritional amount affected fetal mass with the RES ewes having less total fetal mass ($P = 0.01$) and average fetal mass ($P = 0.07$) compared to CON ewes. Gravid uterine mass was reduced ($P = 0.01$) in RES ewes compared to the CON ewes. The fetal:placental mass ratio was greater ($P = 0.06$), in the CON ewes compared to the RES ewes. Diet did not affect placentome number ($P \geq 0.20$) or total placentome weight ($P \geq 0.27$); therefore, average placentome weight was not different among treatments ($P \geq 0.17$). Further, diet had no effect on caruncular or cotyledonary weight ($P \geq 0.24$).

3.2. Maternal progesterone

There was a Se × nutritional level × day three-way interaction on maternal progesterone concentration ($P = 0.01$; Fig. 2). Although Se treatments were initiated before breeding, progesterone concentration did not differ on d 62, prior to initiation of diets with differing nutritional amounts. Progesterone concentrations still did not differ on d 76 but by d 90, the ASe–RES ewes had a greater ($P \leq 0.08$) progesterone concentration compared to all other treatment groups, which did not differ (10.64 ng/mL vs. 7.82, 8.57, and 8.27 ± 0.96 ng/mL for ASe–CON, HSe–CON, and HSe–RES, respectively). By d 104, progesterone concentration in ASe–RES ewes remained elevated ($P \leq 0.003$) and greater than ASe–CON and HSe–CON ewes, which did not differ (14.71 ng/mL vs. 9.71 and 11.12 ± 0.91 ng/mL for ASe–CON and HSe–CON, respectively). The ASe–CON ewes had less progesterone compared to the HSe–RES ewes on d 104 ($P = 0.02$; 9.71 ng/mL vs. 12.69 ± 0.92 ng/mL). By d 118, progesterone concentration was not affected by diet, which remained similar until slaughter.

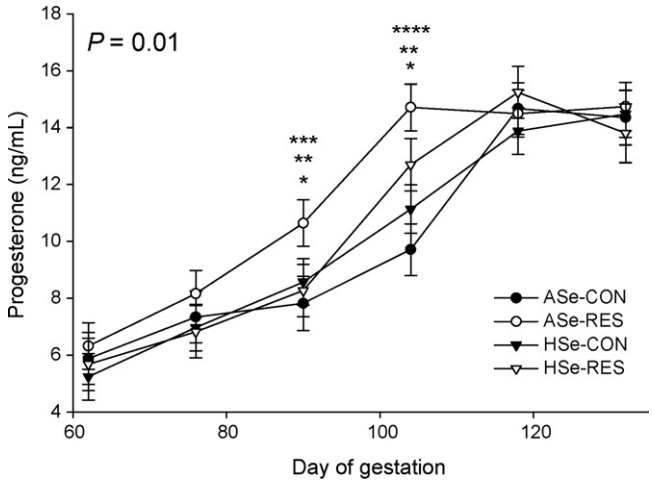

Fig. 2. Effect of dietary selenium (Se) × nutritional amount × day on maternal progesterone concentration in pregnant ewe lambs. ASe = adequate Se ewes (7.4 μg of Se/kg body weight); HSe = high Se ewes (81.5 μg of Se/kg body weight); CON = control ewes fed at 100% requirements; RES = ewes fed at 60% of CON. (*) ASe–CON differs from ASe–RES, $P \leq 0.03$; (**) ASe–RES differs from HSe–RES, $P = 0.06$; (***) ASe–RES differs from HSe–CON, $P \leq 0.08$; (****) ASe–CON differs from HSe–RES, $P = 0.02$.

Table 2
Effect of dietary selenium (Se) and amount of nutrition on estimates of cellularity and proliferating nuclei in maternal and fetal tissues of the placentome.

Item	Selenium[a]		Nutrition[b]		SE	*P*-value		
	ASe	HSe	CON	RES		Se	Nut	Se × Nut
Caruncle								
DNA, mg/g	1.76	1.74	1.62	1.89	0.21	0.94	0.36	0.54
RNA, mg/g	2.95	3.50	2.91	3.54	0.37	0.30	0.22	0.88
RNA:DNA	2.79	2.40	2.80	2.39	0.66	0.67	0.65	0.97
Protein, mg/g	36.82	43.24	41.62	38.45	5.06	0.38	0.65	0.33
Protein:DNA	30.49	28.98	35.17	24.30	7.06	0.88	0.27	0.86
Proliferating cells, %	0.08	0.11	0.12	0.07	0.23	0.41	0.13	0.24
Cotyledon								
DNA, mg/g	3.11	3.91	3.61	3.41	0.31	0.08	0.65	0.38
RNA, mg/g	3.73	4.26	3.73	4.26	0.26	0.15	0.15	0.41
RNA:DNA	1.31	1.16	1.18	1.29	0.10	0.28	0.42	0.77
Protein, mg/g	48.70	45.14	50.96	42.88	3.20	0.44	0.08	0.73
Protein:DNA	15.84	12.42	15.85	12.41	1.28	0.07	0.06	0.44
Proliferating cells, %	1.39	2.78	2.52	1.65	0.46	0.04	0.19	0.18

[a] ASe = 6 μg of Se/kg BW (no added Se); HSe = 80 μg of Se/kg BW (HSe).
[b] CON = control ewes fed at 100% of the NRC (1985) requirements; RES = ewes fed to 60% of CON.

3.3. Placental cellularity

There were no Se × nutritional amount interactions ($P \geq 0.18$) on placental cellularity (Table 2). In caruncular tissue, there was no effect ($P \geq 0.22$) of diet on DNA, RNA or protein concentrations. Further, the RNA:DNA ratio and the protein:DNA ratio in caruncular tissue were not affected by treatment ($P \geq 0.27$). There was no effect of diet on the percentage of proliferating nuclei in caruncular tissue ($P \geq 0.13$). In cotyledonary tissue, neither Se nor nutritional amount affected ($P \geq 0.15$) RNA concentration or RNA:DNA. However, HSe ewes had greater ($P = 0.08$) cotyledonary DNA concentration compared to ASe ewes. Selenium treatment did not affect ($P \geq 0.44$) protein concentration in cotyledonary tissue; however, the ASe ewes had a greater ($P = 0.07$) cell size as indicated by a greater protein:DNA ratio in cotyledonary tissue compared to the HSe ewes. The percentage of proliferating nuclei in the fetal portion of the placenta was affected by Se treatment, with the HSe ewes having a greater percentage ($P = 0.04$) of proliferating nuclei compared to the ASe ewes. Amount of nutrition did not impact ($P \geq 0.65$) DNA concentration in cotyledonary tissue; however, RES ewes had greater ($P = 0.08$) cotyledonary protein concentration compared to the CON ewes. Furthermore, the CON ewes had a greater ($P = 0.06$) cell size in cotyledonary tissue as indicated by a greater protein:DNA ratio compared to the RES ewes. Nutritional amount did not affect ($P \geq 0.19$) the percentage of proliferating nuclei in cotyledonary tissue.

3.4. Vascularity measurements and angiogenic and vasoactive mRNA

There was no effect ($P \geq 0.11$) of Se or nutritional amount on CAD, CND, CSD, or APC in either the caruncular or the cotyledonary tissue (Table 3). There was no Se × nutritional amount interaction on amount of *VEGF*, *KDR*, *Flt*, *eNOS*, or *sGC* mRNA in either caruncular or cotyledonary

Table 3
Effect of dietary selenium (Se) and amount of nutrition on capillary area density (CAD)[a], capillary number density (CND)[b], capillary surface density (CSD)[c], and area per capillary (APC)[d] of the caruncular and cotyledonary tissues.

Item	Selenium[e]		Nutrition[f]		SE	*P*-value		
	ASe	HSe	CON	RES		Se	Nut	Se × Nut
Caruncle								
CAD, %	0.46	0.40	0.41	0.45	0.03	0.18	0.24	0.40
CND, (capillaries/μm^2) × 10^6	2276.19	2541.15	2242.14	2575.21	305.68	0.54	0.44	0.88
CSD, ($\mu m/\mu m^2$) × 1000	133.04	137.77	128.82	141.99	5.63	0.55	0.11	0.64
APC, μm^2	230.06	208.32	219.29	219.09	28.13	0.58	0.99	0.59
Cotyledon								
CAD, %	0.12	0.13	0.12	0.13	0.01	0.52	0.73	0.13
CND, (capillaries/μm^2) × 10^6	1527.98	1592.10	1599.95	1520.13	73.04	0.54	0.46	0.15
CSD, ($\mu m/\mu m^2$) × 1000	69.10	71.17	71.78	68.48	2.58	0.58	0.38	0.75
APC, μm^2	85.31	84.75	80.03	90.03	7.13	0.96	0.34	0.14

[a] Total capillary area (μm^2) as a proportion of tissue area (μm^2).
[b] Total number of capillaries per unit of tissue area (μm^2).
[c] Total capillary circumference (μm) per unit of tissue area (μm^2).
[d] APC = CAD divided by CND.
[e] ASe = 6 μg of Se/kg BW (no added Se); HSe = 80 μg of Se/kg BW (HSe).
[f] CON = control ewes fed at 100% of the NRC (1985) requirements; RES = ewes fed to 60% of CON.

Table 4
Effect of dietary selenium (Se) and amount of nutrition on mRNA concentration of select angiogenic and vasoactive factors and their receptors.

Item	Selenium[a]		Nutrition[b]		SE	*P*-value		
	ASe	HSe	CON	RES		Se	Nut	Se × Nut
Caruncle[c]								
VEGF	21.09	14.95	19.82	16.21	3.98	0.25	0.49	0.88
KDR	17.28	12.04	13.96	15.36	2.62	0.14	0.68	0.76
Flt	10.94	11.96	11.15	11.75	1.41	0.58	0.75	0.31
eNOS	24.77	21.13	22.20	23.69	5.62	0.62	0.84	0.88
sGC	17.78	16.20	16.44	17.54	2.59	0.65	0.75	0.17
Cotyledon[c]								
VEGF	24.27	17.77	24.60	17.43	5.24	0.36	0.31	0.86
KDR	16.18	14.62	15.18	15.62	3.65	0.75	0.93	0.59
Flt	7.27	11.79	7.44	11.63	1.73	0.06	0.08	0.92
eNOS	19.39	20.31	15.98	23.72	6.38	0.91	0.37	0.83
sGC	14.94	15.21	12.38	17.77	3.13	0.95	0.21	0.30

[a] ASe = 6 μg of Se/kg BW (no added Se); HSe = 80 μg of Se/kg BW (HSe).
[b] CON = control ewes fed at 100% of the NRC (1985) requirements; RES = ewes fed to 60% of CON.
[c] Placental angiogenic factor expression expressed as a ratio of angiogenic factor mRNA to 18S.

tissues (Table 4). Further, there was no effect ($P \geq 0.14$) of dietary treatment on amount of *VEGF*, *KDR*, *Flt*, *eNOS*, or *sGC* mRNA in caruncular tissue. While dietary treatment did not affect ($P \geq 0.21$) gene expression of *VEGF*, *KDR*, *eNOS*, or *sGC* in cotyledonary tissue, *Flt* gene expression was altered. Cotyledonary *Flt* gene expression was greater ($P \leq 0.08$) in the HSe ewes compared to ASe ewes and in the RES ewes compared to CON ewes.

3.5. Fetal cortisol, glucose, and glycogen

There was no effect ($P \geq 0.49$) of treatment on fetal serum cortisol or glucose concentrations, which averaged 12.00 ± 0.88 ng/mL and 38.64 ± 1.31 mg/dL, respectively. There was a Se × nutritional amount interaction on fetal liver glycogen concentration ($P = 0.04$) and glycogen per cell ($P = 0.04$; Table 5). Fetuses from the ASe–RES ewes had more ($P = 0.04$) liver glycogen and glycogen per cell compared to fetuses from the ASe–CON and HSe–RES ewes, with fetuses from the HSe–CON ewes being intermediate. There was no effect ($P \geq 0.21$) of treatment on fetal liver DNA which averaged 3.97 ± 0.06 mg/g. Further, when total liver glycogen was calculated, there was no effect of diet ($P \geq 0.11$).

4. Discussion

Reduced fetal mass by supranutritional Se or nutrient restriction is not due to an alteration in placentome number, placentome mass, individual caruncular or cotyledonary weight, or placental vascularity near term. However, placental function or nutrient transport may have been compromised. Cotyledonary tissue appears to be more susceptible to both greater amounts of Se and nutrient restriction than caruncular tissue. This is evidenced by alterations in cellular proliferation, cellularity estimates, and amount of *Flt* mRNA in cotyledonary tissue. In addition, the ASe–RES ewes had increased progesterone from d 90 through d 106 of gestation, and fetuses from the ASe–RES ewes had more liver glycogen concentration compared to the other groups. The alterations to cotyledonary tissue along with greater progesterone concentration and fetal liver glycogen concentration may be due to adaptations of the placenta or the fetus to maternal dietary Se intake and/or nutrient restriction.

In the current study, the CON ewes gained 23.8% of their initial weight throughout the study whereas the RES ewes gained 4.63%. Moreover, the RES ewes had a 15.7% reduction in total fetal mass compared to CON ewes without affecting placental mass. If nutrient restriction occurs earlier in pregnancy (i.e., d 30–96 of gestation), a time frame when maximal placental growth is occurring, placental weight can be reduced at near term without affecting fetal weight (McCrabb et al., 1992a). In the current study, nutrient restriction did not begin until d 64 of gestation, and while the placenta continues to increase in mass until about d 90 of gestation, the majority of growth has already occurred by d 64 of gestation (reviewed by Redmer et al., 2004). Therefore, nutrient restriction during the latter part of pregnancy may not have great impacts on placental mass. Furthermore, in the current study, the RES ewes did not

Table 5
Effect of the Se × nutritional amount interaction on fetal liver glycogen.

Item	Se × Nutrition treatment				SE	*P*-value		
	ASe–CON	ASe–RES	HSe–CON	HSe–RES		Se	Nut	Se × Nut
Liver glycogen, mg/g	33.18a	44.09b	36.07ab	30.12a	4.51	0.17	0.53	0.04
Liver glycogen per cell[a]	8.56a	11.56b	9.11ab	7.44a	1.17	0.12	0.55	0.04
Total liver glycogen[b], g	3.60	3.58	3.81	2.50	0.47	0.29	0.11	0.13

Means ± SE within a row having different letters (a and b) differ ($P < 0.10$).
[a] Fetal liver glycogen concentration (mg/g) divided by fetal liver DNA concentration (mg/g).
[b] Fetal liver weights were previously published (Reed et al., 2007).

differ in body weight from the CON ewes until d 87 of gestation, indicating that even when ewes were restricted, maternal energy stores may have met the nutritional needs for the developing placenta and fetus during this time.

Restriction during late gestation leads to decreased fetal weight. Mellor and Murray (1981) found that 68% nutrient restriction from d 112 to 142 of gestation resulted in reduced fetal weight at d 142, without affecting placental weight. A less severe (i.e., 43%) nutrient restriction from d 100 to term also resulted in a reduced birth weight at term; however, no placental variables were reported (Russel et al., 1977). Rattray and Trigg (1979) found that nutrient restriction from d 95 to 140 of gestation resulted in a reduced fetal weight as well, without a reduction in placental weight. When ewes were restricted from conception until d 90 of gestation, both fetal and placental weight were similar to control ewes; however, when nutrient restriction continued until d 130, fetal weight was reduced in restricted ewes while placental weight was not affected (Luther et al., 2007). Clearly, fetal weight is affected by maternal undernutrition during late gestation.

In the current study, RES ewes have a reduced fetal mass:placental mass ratio compared to the CON ewes, indicating that the reduction in fetal growth occurred while placental weight was spared. Similarly, Vonnahme et al. (2003) found that nutrient restriction between d 28 and 78 of gestation resulted in a reduced fetal mass:placental mass ratio on d 78, where fetal weight was reduced, and placental weight was spared. When ewes were fed a restricted diet to maintain weight throughout gestation and gradually deplete maternal reserves throughout gestation, the fetal mass:placental mass ratio was similar to a control group at both d 90 and 130 of gestation despite a reduction in fetal weight without a reduction in placental weight at d 130 (Luther et al., 2007). In a unique model, Wallace et al. (2002) have shown overnutrition results in IUGR as well. When ewes were fed greater than recommended amounts throughout gestation, both fetal weight and total placentome weight were reduced compared to the control group by d 134 of gestation. However, because fetal weight was relatively less disturbed than placental weight, the fetal mass:placental mass ratio was greater in the ewes fed greater than recommended amounts compared to the control ewes (Wallace et al., 2002).

While few studies have directly looked at how supplemental Se impacts the placenta, it has been reported that IUGR babies have greater Se in umbilical arterial blood compared to appropriate for gestational age controls (Osada et al., 2002). In the current study, HSe ewes had greater fetal weights. We have previously reported that fetal blood from these supplemented ewes has approximately two times the amount of Se compared to the fetuses from control ewes (Ward et al., 2005). Perhaps placental sequestration of Se is an adaptive response in case of fetal stress.

Supranutritional Se reduces certain types of cancer in humans (Clark et al., 1996). Both increased apoptosis and reduced angiogenesis contributed to inhibited tumor growth in rats fed supranutritional Se (Combs and Lu, 2001). During early and mid pregnancy, the placenta is also a rapidly growing tissue (reviewed in Redmer et al., 2004); however, in the present study, greater amounts of Se did not decrease proliferation. In fact, these amounts of Se actually increased cellular proliferation and DNA concentration in cotyledonary tissue while reducing cell size (i.e., protein:DNA ratio). Greater than recommended amounts of dietary Se increase number of cells proliferating in the jejunal mucosa of pregnant ewes (Neville et al., 2008) and in jejunal tissue of beef steers (Soto-Navarro et al., 2004). Both the placenta and small intestine consume a large percentage of maintenance energy in ruminants (Bell, 1993; Caton et al., 2000; Ferrell and Jenkins, 1985; Reynolds et al., 1990b). The mechanism by which Se increases cellular proliferation in these nutrient-transferring and metabolically important tissues is unknown at this time. The role of specific selenoproteins in the jejunum and placenta known to impact oxidative stress needs to be investigated.

While cotyledonary tissue was affected, none of the cellularity estimates, proliferation, or angiogenic factor gene expression in caruncular tissue were affected by either Se intake or nutrient restriction. This indicates that the fetal portion of the placenta is more susceptible to the greater amounts of Se and nutrient restriction. Whereas caruncular vascularity increases via vasodilation throughout gestation, cotyledonary capillary numbers and surface area of nutrient exchange increases tremendously during the last half of gestation (Borowicz et al., 2007). In fact, cotyledonary capillary number density increases at twice the rate of the caruncular tissue during the last two-thirds of gestation (Borowicz et al., 2007), the same time the nutrient restriction occurred in the current study. Unexpectedly, none of the vascularity measures were altered by high Se or nutrient restriction in this study. However, this does not mean placental function was completely spared. In fact, both supranutritional Se and nutrient restriction resulted in increased *Flt* mRNA expression in cotyledonary tissue. While both Flt and KDR are receptors for VEGF, it seems Flt may act as a decoy receptor or by suppressing signaling through KDR, which would suppress angiogenesis (Roberts et al., 2004; Yancopoulos et al., 2000). Moreover, Flt increases vascular permeability (Odorisio et al., 2002; Peters et al., 1999). In cattle, nutrient restriction causes an increase in *Flt* mRNA expression (Vonnahme et al., 2007), which agrees with our findings in ewes. Greater amounts of Se supplementation increased *Flt* mRNA in cotyledonary tissue, which may increase permeability of placental capillary beds, leading to the greater fetal weight observed. However, fetal weight was not spared in the RES ewes despite greater *Flt* gene expression compared to the CON ewes.

Several authors (Dodic et al., 2002; Newnham et al., 1999) have shown that glucocorticoid treatment to the dam leads to IUGR. Further, nutrient restriction increases fetal cortisol concentrations (Rozance et al., 2008). This increased fetal cortisol increases gluconeogenic enzyme activity in the fetal liver (Fowden et al., 1990, 1993). In the model for the present study, greater amounts of Se reduced the relative fetal adrenal weight and resulted in a greater fetal weight (Reed et al., 2007), but neither greater Se nor nutrient restriction affected fetal cortisol concentration in the current study. Adrenal gland size may not be a good indicator of circulating levels of cortisol in the fetus, but may lead to differing metabolism in offspring postnatally.

The impacts of Se on adrenal gland function and postnatal metabolism need to be further investigated.

The lack of differences in fetal glucose does not reflect the reduced amount of maternal nutrition in the RES ewes. Perhaps the similar glucose levels are due to differential gluconeogenic rates. During times of fasting, the ovine fetus relies on glucose production through gluconeogenesis (Dalinghaus et al., 1991). Limesand et al. (2007) imposed high temperatures on ewes to produce a model of placental insufficiency and found that fetuses from the heat-stressed ewes had increased mRNA for hepatic gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P). When ewes were fasted from d 120 to 125 of gestation specific activity of both PEPCK and G6P in fetal livers was increased compared to control ewes on d 125 of gestation (Lemons et al., 1986). These data indicate that IUGR fetuses are enzymatically capable of gluconeogenesis. In the present study, the RES fetuses had similar amounts of glucose compared to CON fetuses, despite maternal nutrient restriction. Glucose demands in the RES fetuses may have been met through increased gluconeogenesis, which is a possible explanation why fetal glucose concentrations did not differ among treatments. We have previously reported that fetal liver masses from the RES ewes were lighter compared to CON ewes, while Se had no influence on fetal liver mass (Reed et al., 2007). Despite these differences in fetal liver masses, total glycogen content did not differ. Gluconeogenesis is dependent upon nonglycosidic stores, primarily lactate and amino acids in the fetal liver of sheep. When nutrient supply was restricted to 50% of maintenance requirements from d 28 to 78 of gestation, fetal weight was reduced without affecting placental weight (Vonnahme et al., 2003). Furthermore, concentrations of amino acids, particularly serine and arginine families, in maternal and fetal plasma were reduced in restricted ewes compared with controls (Kwon et al., 2004). In the current study, amino acid concentrations were not determined; however, it would be useful to measure amino acid concentration in future studies using a similar model to the current study to determine if nutrient restriction is altering fetal amino acid concentrations. Greater fetal liver glycogen and liver glycogen per cell, as observed in the ASe–RES ewes in the present study, may help ensure neonatal survival.

Progesterone concentrations were similar among treatment groups until d 90 of gestation, at which time ASe–RES ewes had an increase in progesterone compared to all other groups. Progesterone continued to be elevated in ASe–RES ewes compared to the ASe–CON and HSe–CON groups through d 106 of gestation. After this time, all treatment groups had similar concentrations of progesterone until the end of the study. As relative liver weight to ewe body weight did not differ for the ewes in this study (Reed et al., 2007), differences in progesterone concentration may be associated with placental growth. The increases in circulating progesterone measured from d 62 to 106 in the ASe–RES ewes may be due to increased placental growth during that time. When ewes were underfed from d 30 to 96 of gestation placental weight was greater compared to control ewes at d 96 of gestation (McCrabb et al., 1992b). Because placental mass was only measured near term, it is unknown how placental growth progressed.

In summary, both the fetus and the placenta may have adapted to greater maternal Se intake and/or nutrient restriction to ensure fetal survival as evidenced by changes in fetal liver glycogen concentration, as well as placental cellularity, progesterone production, and amounts of *Flt* mRNA.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the research reported.

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